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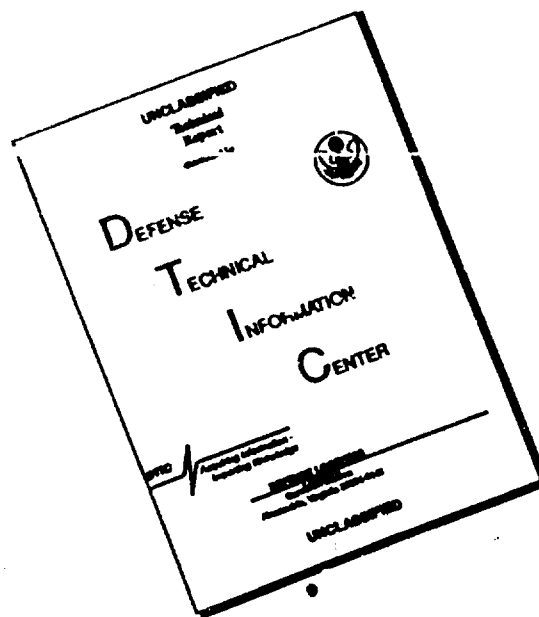
THE USE OF THE METHOD OF REDUCTION OF METHYLENE  
BLUE FOR THE RAPID DETERMINATION OF THE LIVING  
BACTERIA COUNT WHEN GROWING THE PLAGUE MICROBE BY  
THE DEPTH METHOD IN THE REACTION TANK

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15 October 1965

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THE USE OF THE METHOD OF REDUCTION OF METHYLENE BLUE  
FOR THE RAPID DETERMINATION OF THE LIVING BACTERIA COUNT  
WHEN GROWING THE PLAGUE MICROBE BY THE DEPTH METHOD IN THE  
REACTION TANK.

By  
I. V. Liskina (Saratov)  
pp. 269-274.

In the preceding work we showed (1960) that the method developed by us for the determination of living microbes in the anti-plague vaccine, based on the rate of reduction of methylene blue by the plague microbe, enables us to determine rapidly and with sufficient accuracy the living microbe count in the vaccine suspension according to the time of discoloration of the pigment.

In the present study we used the above-mentioned method for the control of the living microbe count in the process of cultivation of the vaccine strain of plague microbe by the depth method in the reaction tank.

As a material for the research we used samples of the microbe slurry taken from the reaction tank in which the YeV anti-plague vaccine was grown for the development of technological rules of the production of dry living vaccine, conducted by the collective of workers of the "Microbe" Institute under the guidance of Prof. N. I. Nikolayev.

The culture of the plague microbe (the YeV strain) was grown 24-72 hours in 500-liter reaction tanks at 26-28°C with aeration and mechanical mixing of the culture medium (360 rpm).

The inoculum was the 22-hour culture, grown on the Huttiger bouillon under aeration condition. Per 1 milliliter of the nutrient medium not less than 300 million microbe cells were introduced, according to the GKI [Gosudarstvennyy Kontrol'nyy Institut Meditsinskikh Biologicheskikh Preparatov im. L. A. Tarasevicha; State Control Institute of Medical Biological Preparations imeni L. A. Tarasevich -- SCI] standard of cloudiness.

Every three hours samples were taken from the reaction tank, and in these samples the pH, optical and biological microbe concentration and the reduction time of methylene blue

were determined. Each sample was controlled for the net growth by examining the Gram-tinted smears, as well as sowing on control nutrient media (agar plates, bevelled agar, and semi-liquid agar). The biological concentration of the slurry was determined by the cultural method adopted in the production. The estimate of time of reduction of methylene blue by the microbe slurry is described in the preceding work.

By the above-mentioned methods we performed a relative determination of the living microbe count in growing of the plague microbe in Huttinger's bouillon; in the same bouillon, but with an additional nutrition with glucose; and in the bouillon made from casein fermentation hydrolyzate.

We have shown that in the process of growth of the plague microbe in Huttinger's bouillon there is a direct correspondence between the discoloration rate of methylene blue and the living microbe count in their determination by the cultural method (Fig. 1).

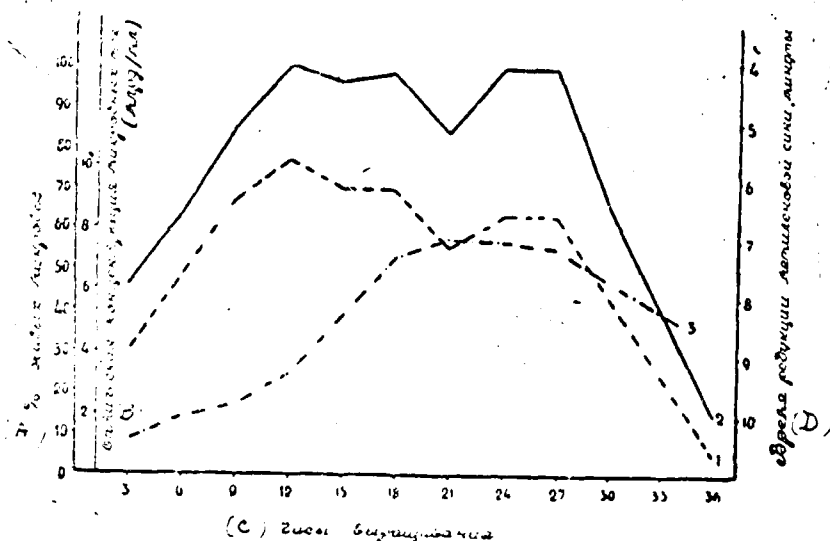


Fig. 1. Dynamics of the growth of plague microbe in Huttinger's bouillon under the conditions of depth aeration in a reaction tank.

1 -- discoloration time of methylene blue; 2 -- living microbe count; 3 -- optical concentration of microbe bodies.

LEGEND: (A) % of living microbes; (B) optical concentration of microbe bodies (billion/milliliter) (C) hours of cultivation; (D) Reduction time of methylene blue, minutes.

On the basis of the above we compiled a standard scale showing the correspondence between the discoloration

time of methylene blue and the living microbe count, determined by the cultural method, which enabled us to estimate the living microbe contents in individual samples from the reaction tank by the time of discoloration of methylene blue only.

In subsequent experiments we verified the feasibility of the method proposed by us in the cultivation of the plague microbe in Huttinger's bouillon prepared with a phosphate buffer with the additional glucose nutrition of the culture in the growing process. The glucose was added every hour in 75 ml portions of the 40% solution, or continuously, 30-60 drops per minute by means of a dropper built-in the reaction tank according to M. V. Altukhov's method. In the first, intermittent, addition of glucose to the volume of the nutrient medium a total of 1% glucose was introduced during the entire growing period, in the second, drop method, -- 0.5-1%. Taking into consideration that the additional nutrition with glucose at times resulted in a considerable lowering of pH (below 7.0) the latter was rigidly controlled.

Since glucose is dehydrated by the plague microbe, it was important to determine its effect in the additional nutrition of the culture, on the relationship established by us in the indications of living microbe counts in the samples, determined by the cultural method and according to the reduction time of methylene blue.

In Fig. 2 we adduce results of the experiment in which glucose was added by the drop method after 24 hours of cultivation. No change in the reaction of the medium toward acidity was observed during the growing of the culture (pH = 7.3-7.9), which indicated the complete utilization of the glucose added. In samples taken before 36 hours of culture growth, there was a full correspondence between the living microbe count determined by the cultural method and according to the reduction time of methylene blue. However, after that time a larger living microbe count began to be determined, than according to the discoloration time of methylene blue.

The cause for this may be in the fact that microbes after 30-36 hours of growth become appreciably smaller than in preceding samples. Determining the concentration of such microbes according to the SCI general-purpose standard of cloudiness, we actually lowered the true microbe count in the given samples, since in the suspension with the same degree of cloudiness there are always more small microbes than there are large ones (Fikhsman, 1960).

Thus we permitted an inaccuracy in the computation of the percentage of living microbes in the slurry, increasing it accordingly (the percentage of living microbes is the ratio of the number of colonies grown to the total number of sown microbes, determined by the standard of cloudiness). Indeed, in samples from the reaction tank after 30-36 hours of cultivation of the plague microbe, a very high living

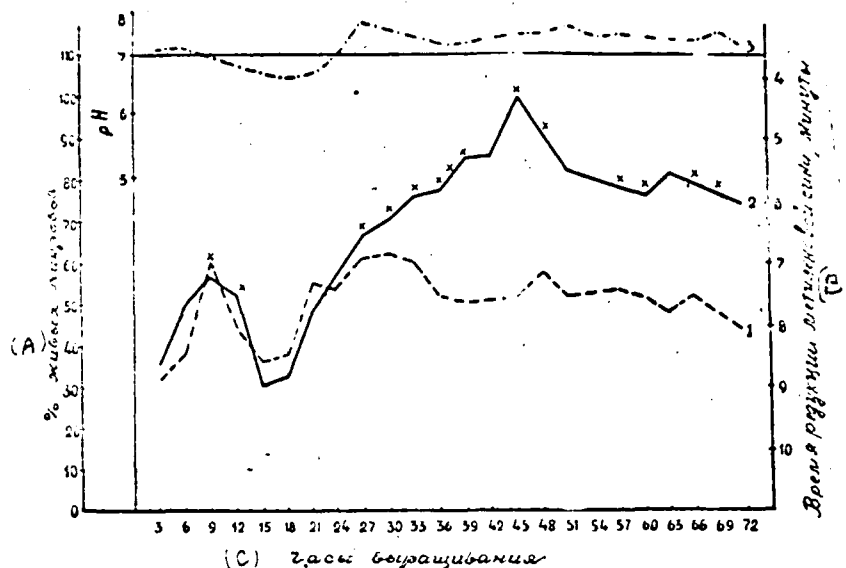


Fig. 2. Dynamics of the Plague Microbe Growth in Hattinger's Bouillon With Additional Glucose Nutrition by the Intermittent Method Under Aeration Conditions in the Reaction Tank.  
1 -- Reduction time of methylene blue; 2 -- living microbe count; 3 -- pH of the microbe suspension; x -- addition of glucose.  
LEGEND (A) % of living microbes; (B) Reduction time of Methylene blue, minutes; (C) Hours of cultivation.

microbe count, -- 100%, and more frequently -- 130-140%, was determined by the cultural method. This error of the cultural method may be avoided if in these hours of growth the SCI standard of cloudiness for smaller particle size were used. In this instance the correspondence between the discoloration time of methylene blue and the living microbe count, determined by the sowings, would be observed in the course of the entire cultivation time of the plague microbe in the reaction tank. On the other hand, the discoloration time of methylene blue in the same samples did not depend on the inaccuracy of determination of the optical concentration, since it was always performed with a slurry diluted to 1 billion according to the standard of cloudiness. If in this suspension a somewhat larger amount of microbes (1.3-1.4 billion) was present, this divergence in the concentration did not have a noticeable effect on the discoloration rate of methylene blue.

As we have already noted, the difference between the results of determination of the living microbe count in the slurry by the one and the other method is exposed only after

36 hours of the growth of the culture; now, taking into consideration that under industrial conditions the anti-plague vaccine is today cultivated for only 18-20 hours, this discrepancy does not decrease the practical significance of the method of determination of living microbes according to the reduction of methylene blue.

Experiments on the growth of the plague microbe in Huttlinger's bouillon with the additional glucose nutrition (by either method) have shown, that the addition of glucose did not have any effect on the results of determination of the living cell count by the cultural method and according to the time of discoloration of the pigment during the first 36 hours of growth.

However, we should bear in mind that an excessive addition of glucose at the beginning of the growth of the culture results in a sharp lowering of pH of the culture medium and, hence, in a considerable dying off of the microbe cells. In such instances the correspondence between the living microbe count and the discoloration time of methylene blue is upset. As we see in Fig. 3, in the samples taken from the reaction tank between 9 and 24 hours of cultivation, the living cell count, determined by the cultural method, remained low (under

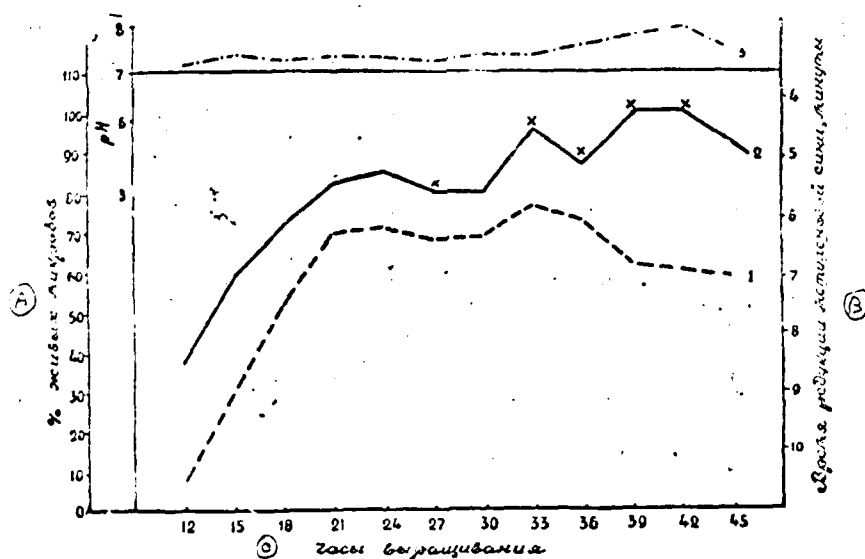


Fig. 3. Dynamics of Growth of the Plague Microbe in Huttlinger's Bouillon With Additional Glucose Nutrition by the Drop Method in a Reaction Tank Under Aeration Conditions.

1 -- reduction time of methylene blue; 2 -- living microbe count; 3 -- pH of the microbe suspension; x -- addition of glucose.  
LEGEND: (A) % of living microbes; (B) Reduction time of methylene blue, minutes; (C) hours of cultivation.



90.6), whereas the methylene blue was reduced much faster. This indicated the incomplete utilization of glucose in the given samples, which speeded up the process of discoloration of methylene blue. After 24 hours of cultivation, the added glucose had no effect on the process of reduction of the pigment; it was evidently fully utilized by the plague microbe (pH 7.3-7.4).

Thus the addition of glucose in the process of growth of the plague microbe should be performed with a rigid dosage of its amounts, continuously checking the reaction of the culture medium and only after the latter has changed toward the alkaline (pH 7.3-7.4) through the propagation of microbes.

In growing the plague microbe in the reaction tank in the culture medium of casein fermentation bouillon with an additional glucose nutrition and without it, the direct correspondence was also manifest between the reduction rate of methylene blue and the living cell content, determined by the cultural method (Fig. 4).

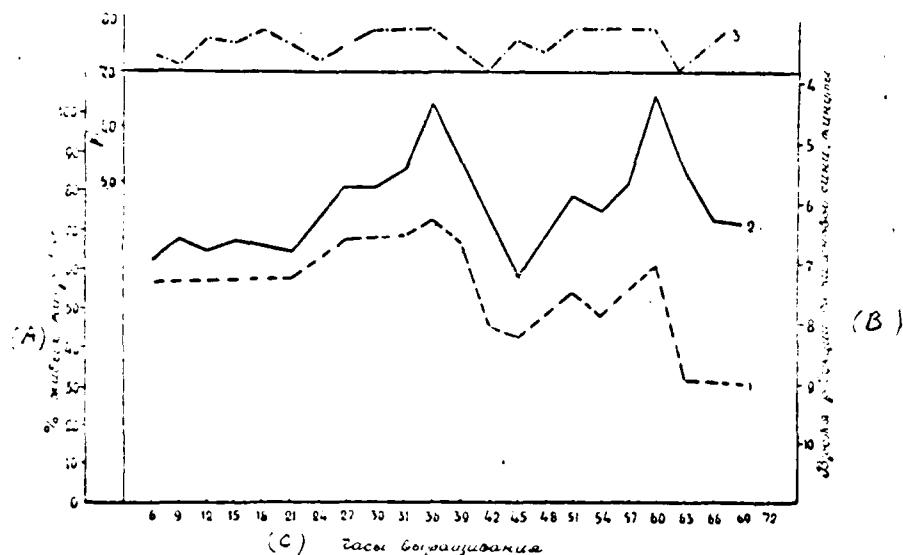


Fig. 4. Dynamics of the Growth of Plague Microbe in the Casein Fermentation Bouillon in the Reaction Tank Under the Conditions of Depth Aeration.

1 -- Reduction time of methylene blue; 2 -- living microbe count; 3 -- pH of the microbe slurry.

Legend: (A) % of living microbes; (B) Reduction time of methylene blue, minutes; (C) Hours of cultivation.

Consequently, the method of reduction of methylene blue can be used under industrial conditions for the determination of the living microbe count in the process of cultivation of the vaccine strain of the plague microbe. Thereby it becomes possible to control the cultivation time, necessary for the production of the largest amount of biomass when using various nutrient media under the conditions of depth growth in reaction tanks.

#### CONCLUSIONS

1. There is a direct correspondence between the living microbe count determined by the cultural method, and the discoloration rate of methylene blue at individual phases of growth of the plague microbe in the reaction tank.

2. The method of reduction of methylene blue can be used for the rapid determination of living microbes in the process of growth of the plague microbe in a reaction tank under the aeration conditions. This enables us to control the time necessary for obtaining the largest yield of the biomass.

3. The additional nutrition of the plague microbe with glucose in the process of growth of the plague microbe, which results in a sharp decrease of pH of the culture medium, disrupts the established direct correspondence between the discoloration rate of methylene blue and the living microbe count, determined by the cultural method.

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